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Discovery of oligosaccharide antigens for semi-synthetic glycoconjugate vaccine leads against *Streptococcus suis* serotypes 2, 3, 9 and 14

Shuo Zhang[a][b]§, Mauro Sella[a]§, Julinton Sianturi[a]§, Patricia Priegue[a][b], Dacheng Shen[a][c] and Peter H. Seeberger[a][b]*

[a] Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14476 Potsdam, Germany

[b] Institute of Chemistry and Biochemistry, Freie Universität Berlin, Arnimallee 22, 14195 Berlin, Germany

[c] Present address: Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA

§ These authors contributed equally

* Corresponding author

Abstract

Streptococcus suis bacteria are one of the most serious health problems for pigs and an emerging zoonotic agent in humans working in the swine industry. *S. suis* bacteria express capsular polysaccharides (CPS) a major bacterial virulence factor that define the serotypes. Oligosaccharides resembling the CPS of *S. suis* serotypes 2, 3, 9, and 14 have been synthesized, glycans related to serotypes 2 and 9 were placed on glycan array surfaces to screen blood from infected pigs. Lead antigens for the development of semi-synthetic *S. suis* serotype 2 and 9 glycoconjugate veterinary vaccines were identified in this way.

Introduction

Streptococcus suis causes bacterial infections in farm pigs globally,[1] but is also a commensal bacterium that commonly inhabits the upper respiratory, digestive and reproductive systems of pigs.[2,3] Virulent strains can infect the bloodstream and eventually result in septic shock and meningitis in pigs, but can also cause septicemia and meningitis in humans.

S. suis is surrounded by a layer of polysaccharides forming the bacterial capsules that play a fundamental role for pathogen survival,[4] protect the bacterium and are important virulence factors. CPSs are able to trigger an adaptive immune response resulting in the production of specific antibodies rendering polysaccharides attractive targets for antibacterial vaccine development.[5] *S. suis* serotypes are distinguished based on the chemical composition of the

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capsules.[6,7] Serotypes 1/2, 2, 3, 7, 9 are most frequently isolated from infected animals and differ in geographical prevalence. Serotype 2 is particularly frequent in Europe and Asia, while serotypes 3 and 9 were mostly found in North America.

Vetenary vaccines are an effective strategy to limit disease in farm animals and reduce the spread of pathogens between animals and transmission to humans. Vaccinations help to reduce antibiotic consumption and slow the development of antimicrobial resistance.[8] All currently used antibacterial vetenary vaccines are prepared from live attenuated or inactivated bacteria that suffer shortcomings in terms of safety, stability and in some cases limited immunogenicity.[8–10] While glycoconjugate vaccines in humans are very successful, veterinary glycoconjugate vaccines remain a largely unexplored opportunity.[11]

The CPS structures of four major *S. suis* serotypes (2, 3, 9, and 14) have been elucidated (Figure 1).[12–15] The CPSs include rare sugars, a variety of glycosidic linkages, anionic charges and modifications such as acetyl esters and phosphodiesters. Serotypes 1, 2, and 14 are structurally very similar.

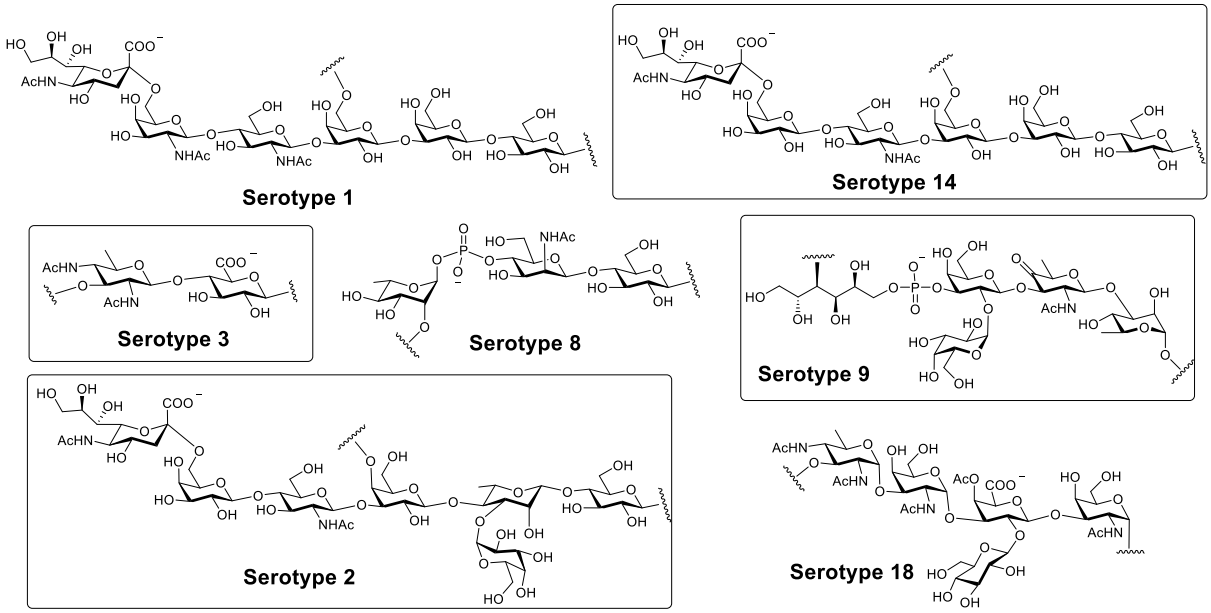

Figure 1. Structures of the most common *S. suis* CPS repeating units. Highlighted structures were the focus of the studies disclosed here.

S. suis vaccine development using isolated polysaccharides has focused exclusively on serotype 2.[16] To date, no single antigen has been shown to be more efficacious than a suspension of killed or weakened bacteria,[16–18] or to be protective against *S. suis* serotype 9. Exact carbohydrate epitopes responsible for inducing protective antibodies are still unknown but are the basis for establishing structure-immunogenicity relationships for the design of carbohydrate antigens for vaccine studies. Investigations with isolated native CPSs produced inconclusive results.[19–21] Synthetic oligosaccharides related to CPS can help to determine antibody epitopes.

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Here, we describe the first synthesis of well-defined oligosaccharides resembling the *S. suis* serotypes 2, 3, 9 and 14 CPSs. Glycans related to serotypes 2 and 9 were employed on the surface of glycan arrays to identify lead structures for the development of semi-synthetic glycoconjugate vaccines against *S. suis*.

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Synthesis of oligosaccharides related to *S. suis* serotype 2 CPS

The *S. suis* serotype 2 CPS[14] consists of a branched heptasaccharide repeating unit ([→4)[α-Neu5Ac(2→6)-β-D-Gal(1→4)-β-D-GlcNAc(1→3)]-β-D-Gal(1→4)-[α-D-Gal(1→3)]-β-L-Rha(1→4)-β-D-Glc(1→]) (Figure 2). The CPS from *S. suis* serotype 2 is essential for its virulence as it prevents phagocytosis when the bacterium infiltrates the bloodstream.[22]

The CPS is the most promising antigen[23,24] as it can induce protective IgM antibodies[25,26] despite being poorly immunogenic ─ low levels of anti-CPS antibodies were seen in pigs after infection[18] or immunization.[23] An anti-serotype 2 glycoconjugate vaccine made from capsular polysaccharides isolated from fermented bacteria was evaluated in immunization experiments in animal models.[16] The poor immunogenicity of CPS can be overcome and protection against *S. suis* can be achieved by active immunization with a glycoconjugate.

Five oligosaccharides (**1**-**5**) resembling the repeating unit of *S. suis* serotype 2 CPS were designed, to obtain detailed structural information of antigenic epitopes of antibodies from *S. suis*-infected pigs (Figure 2). Three shorter fragments were included: trisaccharide **1** resembles the backbone while **2** and **3** represent the side-chain. Pentasaccharide **4** and hexasaccharide **5** were synthesized to cover almost the entire length of a repeating unit as well as branched sequences, and to understand whether the terminal *N*-acetyl neuraminic acid is directly engaged in antibody binding. All synthetic oligosaccharides contain an aminopentyl spacer at the reducing end sugar for creating microarrays and protein conjugates.

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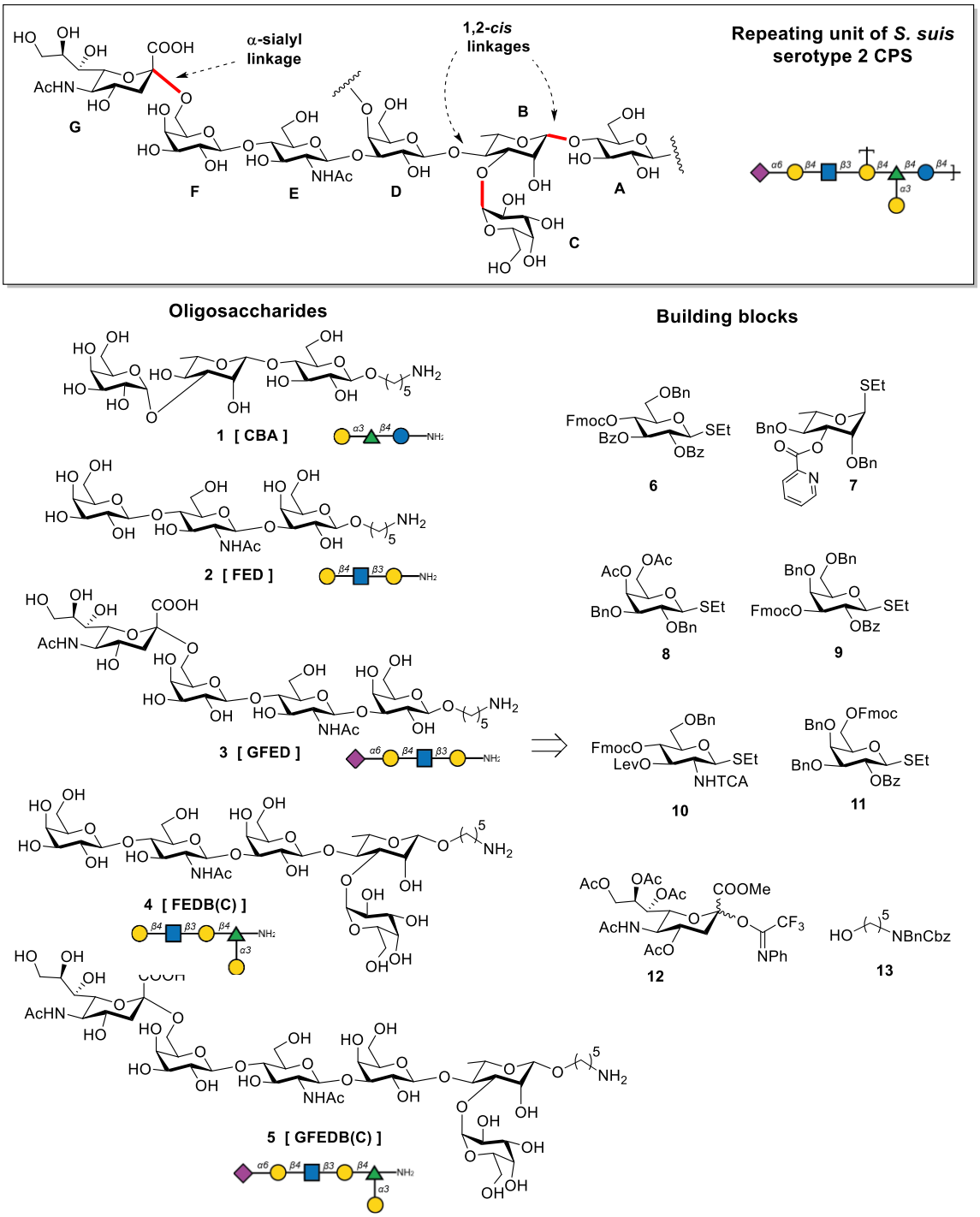

Figure 2. Structure of *S. suis* serotype 2 CPS and related oligosaccharides to be prepared from building blocks **6**–**12**.

Seven orthogonally protected monosaccharide building blocks **6**–**12** were identified to create 1,2-*cis* glycosidic bonds, a branching point on L-rhamnose and the *α*-sialyl linkage (Figure 2). The synthesis of **1** via a linear approach used three monosaccharide building blocks (**6**-**8**) (Scheme 1) and started with the introduction of the spacer at the reducing end monosaccharide with a glycosylation between *N*-protected aminopentanol **13** and glucose thioglycoside **6**, followed by cleavage of the fluorenylmethoxycarbonyl (Fmoc) protective group to obtain **14**. The reducing end glucose was then glycosylated with rhamnose thioglycoside **7**.[27] To assist

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the formation of the β-rhamnosidic linkage and prepare for the subsequent introduction of the α-galactose, the C-3 hydroxyl group is masked by a 2-pyridinecarbonyl ester (picoloyl ester – Pico) that ensures a H-bond mediated stereodirecting effect and is orthogonal to the benzyl ethers. The non-reducing end galactose was introduced with α configuration using known galactosyl thioglycoside **8**,[28] equipped with C-4 and C-6 acetyl esters to assist in the formation of the 1,2-*cis* glycosidic bond. The glycosylation of disaccharide **15** with thioglycoside **8** was carried out in a DCM/Et_2O mixture to increase the α-selectivity through solvent effects. Only the α-linked product **16** was detected on TLC and isolated. Finally, protected trisaccharide **16** was fully deprotected by ester hydrolysis using sodium methoxide in methanol followed by catalytic hydrogenation, obtaining trisaccharide **1**.

The linear synthesis of trisaccharide **2** used commercially available galactose building block **9** as starting point for both monosaccharides **17** and **20** (Supporting information). Spacer **13** was regioselectively glycosylated with **17,** before the resulting galactose was glycosylated with commercially available glucosamine **10**, furnishing disaccharide **19a** after Fmoc removal (Scheme 1). The disaccharide was finally glycosylated with galactose **20** to obtain fully protected trisaccharide **21**. Deacylation with sodium methoxide in methanol at 35 °C was accompanied by partial hydrolysis of the trichloroacetamide due to the large excess of base such that *N*-acetylation became necessary. Finally, catalytic hydrogenation removed all ethers and afforded deprotected trisaccharide **2**.

A convergent 2+2 glycosylation strategy was followed to assemble tetrasaccharide **3**, by first coupling acceptor **19a** and disaccharide **23** that contains a preinstalled α sialyl glycosydic bond. Disaccharide **23** was obtained by glycosylating galactose acceptor **22** with known sialyl glycosyl imidate **12**[29] at –60 °C in a DCM/CH_3CN mixture. Isolation of the pure diastereoisomers by careful silica column chromatography gave pure α-sialylated galactoside **23** and the corresponding β–isomer in a 6/1 α/β ratio. The configuration was unequivocally determined by measuring the long-range $J_{C\text{-}1,H\text{-}3ax}$ (Supporting information).

Disaccharide **23** was employed to glycosylate acceptor **19a**, promoted by NIS and triflic acid. These conditions proved ineffective for tetrasaccharide formation. To improve the reactivity of the glycosylating agent, the thioglycoside was converted to the more reactive glycosyl phosphate **24.** The glycosylation of acceptor **19a** using disaccharide **24** proceeded poorly as no product was isolated from a complex mixture. Likely, insufficient acceptor nucleophilicity was responsible for the failure of this reaction as both glycosylating agents **23** and **24** hydrolysed but acceptor **22** was recovered. The C-4 hydroxyl group in ester protected glucosamine acceptors is a poor nucleophile[30] and variations in the protecting group pattern can lead to

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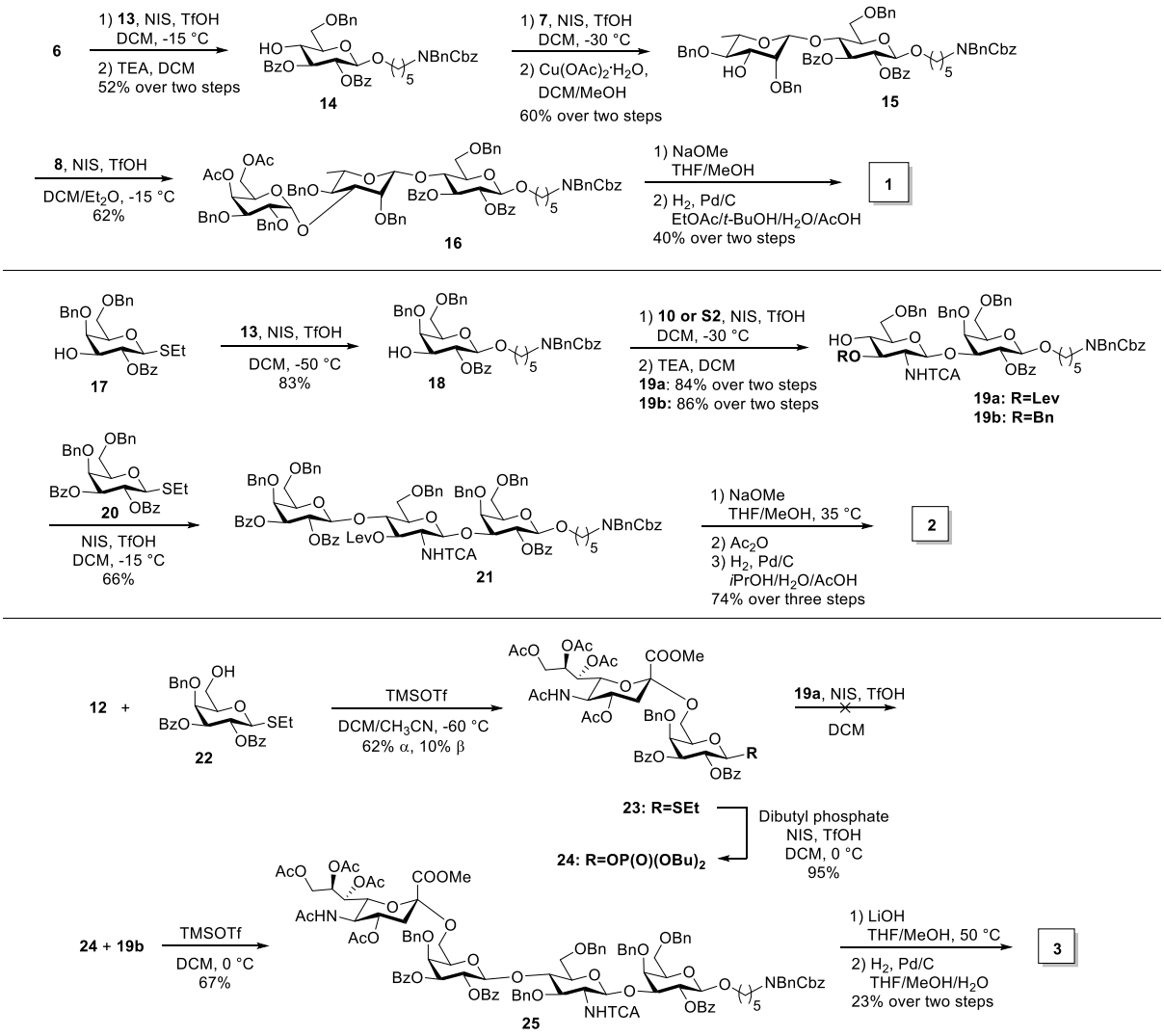
improved couplings. When disaccharide **19b**, containing an ether group instead of an ester at C-3 of the glucosamine unit was glycosylated using disaccharide phosphate **24**, the desired tetrasaccharide **25** was obtained in 67% yield. Removal of all protecting groups by ester hydrolysis under basic conditions followed by catalytic hydrogenation gave unprotected tetrasaccharide **3**.



Scheme 1. Synthesis of linear oligosaccharides **1**, **2** and **3**

Oligosaccharides **4** and **5** were assembled using convergent syntheses from common trisaccharide acceptor **29** (Scheme 2). Trisaccharide **29** contains a challenging β-rhamnosidic linkage on a doubly substituted terminal rhamnose residue. Building block **7** was not suitable for the synthesis and fully orthogonal rhamnose building block **26** was prepared instead in seven steps from commercially available rhamnose (Supporting information, Scheme S2). A glycosylation of the aminopentanol linker using rhamnose tioglycoside **26** and NIS/TfOH as activator resulted in low stereoselectivity (2.4/1, β/α) as judged by NMR. Instead, bromine activation[31] of thioglycoside **26** afforded spacer-linked rhamnose in a slow reaction with

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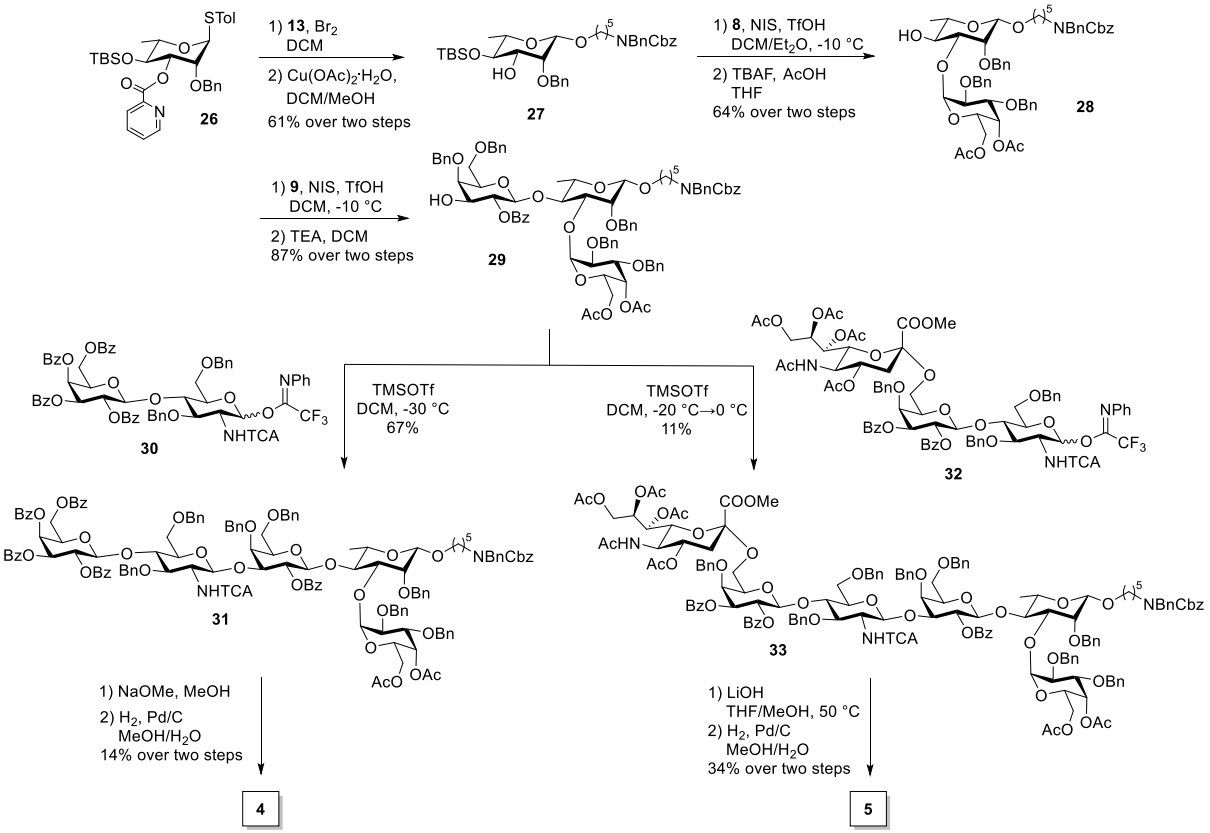
higher stereoselectivity (10/1, β/α). Rhamnose **27** was then glycosylated with galactose **8** in a DCM/Et_2O mixture and no appreciable amounts of β-linked galactose were isolated. To perform a second glycosylation and install a second galactose residue, the silyl ether was removed by TBAF to afford disaccharide acceptor **28** that was glycosylated with **9** to obtain desired trisaccharide **29** after Fmoc removal. The C-4 hydroxyl of rhamnose acted as a good nucleophile, despite its proximity to the α galactose unit.

With trisaccharide acceptor **29** in hand, the assembly of **4** continued with the preparation of disaccharide imidate **30** (Scheme 2 and Supporting information, Scheme S3). Disaccharide **30** and acceptor **29** were coupled to obtain the protected pentasaccharide in 67% yield. Removal of all protective groups yielded pure pentasaccharide **4**.

Sialylated hexasaccharide **5** required the preparation of trisaccharide glycosyl imidate **32** (Scheme 2 and Supporting information, Scheme S4). A 3+3 glycosylation of trisaccharide imidate **32** and trisaccharide **29** did not proceed below –20 °C as **32** degraded. Increasing amounts of acid activator (up to 0.5 eq) and higher temperatures still resulted in highly complex mixtures as only 11% of hexasaccharide **31** was obtained. An identical acceptor was successfully employed in the synthesis of oligosaccharide **4.** Therefore, likely trisaccharide glycosylating agent **32** is responsible for the low yields although the reasons remained unclear. Subsequently, ester cleavage and catalytic hydrogenation produced hexasaccharide **5**.



Scheme 2. Synthesis of branched oligosaccharides **4** and **5**

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S. suis serotype 2 pentasaccharide is a potential vaccine candidate

Glycan arrays enable the screening of multiple serum samples to identify protective glycotopes.[32] Synthetic oligosaccharides **1**-**5**, the isolated native CPS, and other structurally related glycans were immobilized on glass slides to identify antibody binding patterns in serum samples from infected pigs and from rabbits immunized with native CPS (Figure 3). In addition, human reference serum 007sp was screened as control.[33]

IgG antibodies from pig sera bound specifically to oligosaccharide **1** and, to a lesser extent, to **4**. A five-fold stronger binding to oligosaccharides **4** and **5** was observed with anti-serotype 2 rabbit serum (Figure 3). Co-infections with other *S. suis* serotypes or bacteria in pigs may be responsible for these differences as higher antibody levels are elicited following immunizations compared to a natural infection.[34]

Trisaccharide **1** was recognized not only by pig sera, but also by *S. pneumoniae*-vaccinated human serum (007sp). This finding is likely the result of immune cross-reaction due to structural similarities in the CPS from different streptococcal species.[35,36]

Pentasaccharide **4**, a structure that covers most of the native repeating unit, appears to be the minimum glycotope useful to elicit an immune response, as both pig and rabbit antibodies recognized it strongly. It is worth noting that almost identical fluorescence signals were measured for compounds **4** and **5**. The terminal sialic acid unit on hexasaccharide **5** neither increased nor impaired binding to these antibodies. Therefore, sialic acid is not a fundamental part of the minimal epitope of rabbit antibodies.

Additionally, class switch from IgM to IgG antibodies occurred as IgG titres were found to be much higher than the IgM response (Supporting information, Figure S2), likely as result of B cell differentiation to eliminate the pathogen following immunization or infection.[16] Immunization resulted in higher antibody titres than natural infections,[34] suggesting that vaccination with glycoconjugates is a promising means to elicit an even stronger immune response. In conclusion, pentasaccharide **4** is an attractive lead for the development of a glycoconjugate vaccine to protect from *S. suis* serotype 2.

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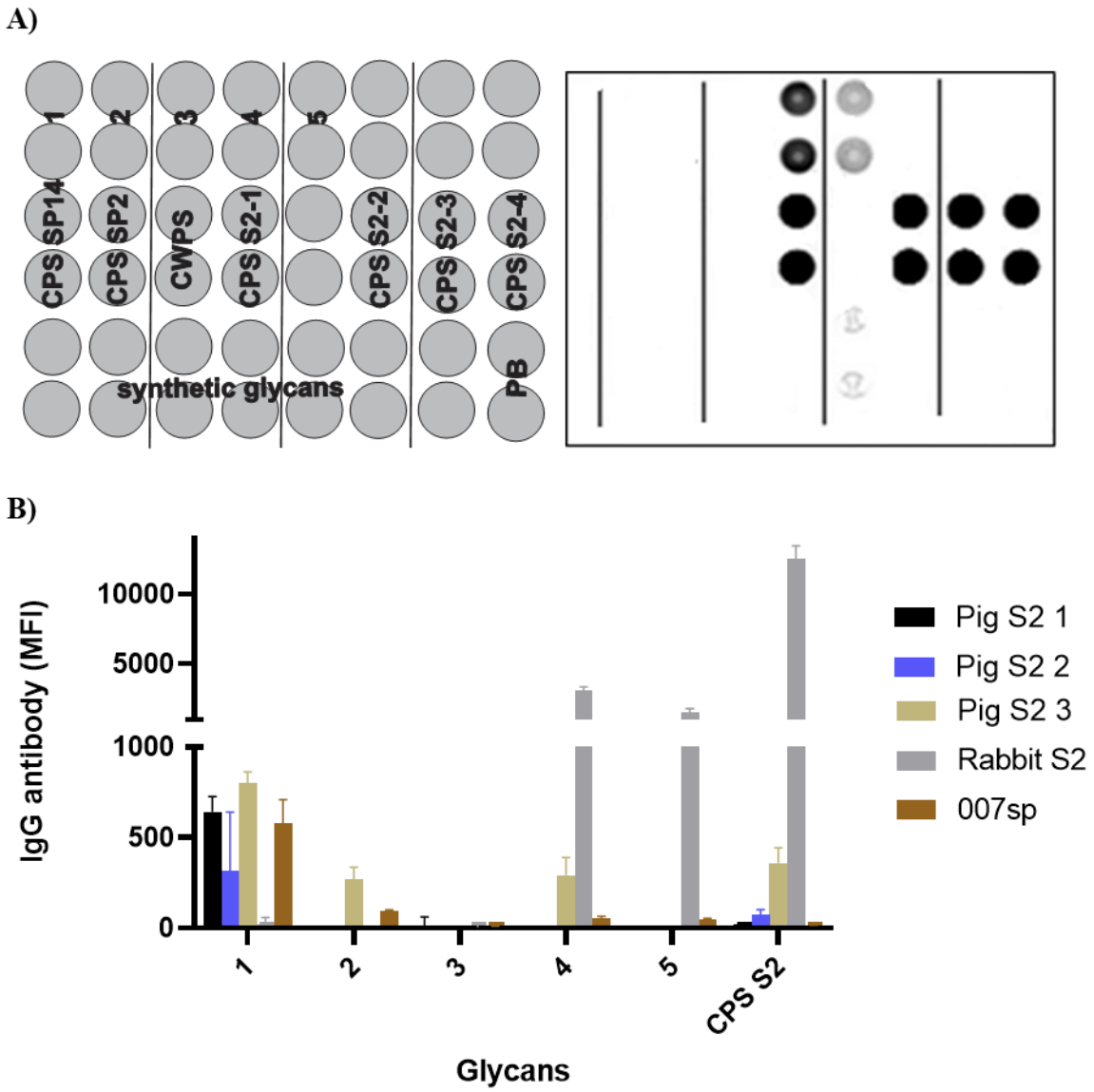

Figure 3. Glycan array analysis of *S. suis* serotype 2 oligosaccharides and native CPS. A) Printing pattern of microarray and binding pattern of rabbit serum to immobilized glycans. B) IgG antibody binding to glycans. A serum dilution of 1:100 was used. MFI, mean fluorescence in intensity (mean ± standard deviation); PB, printing buffer; CWPS, cell wall polysaccharide; CPS and synthetic glycans, see Figure S1.

Synthesis of oligosaccharides related to *S. suis* serotype 3 CPS

The *S. suis* serotype 3 CPS disaccharide repeating unit [→4)-*β*-D-Glc*p*A-(1→3)-*β*-D-Qui*p*NAc4NAc-(1→] contains the rare diamino sugar di-*N*-acetyl-D-bacillosamine (Qui*p*NAc4NAc) and D-glucuronic acid (GlcA) (Figure 4).[12] Qui*p*NAc4NAc is found in various bacterial capsular polysaccharides, including *N*-linked glycoproteins on *Campylobacter jejuni*, *O*-linked glycoproteins on *Neisseria gonorrhoeae* and O-antigens from many strains of

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Gram-negative bacteria.[37–39] The *S. suis* disaccharide repeating unit is unique and we report the first synthesis of a series of oligosaccharides related to the native *S. suis* serotype 3 CPS as basis for immunological studies.

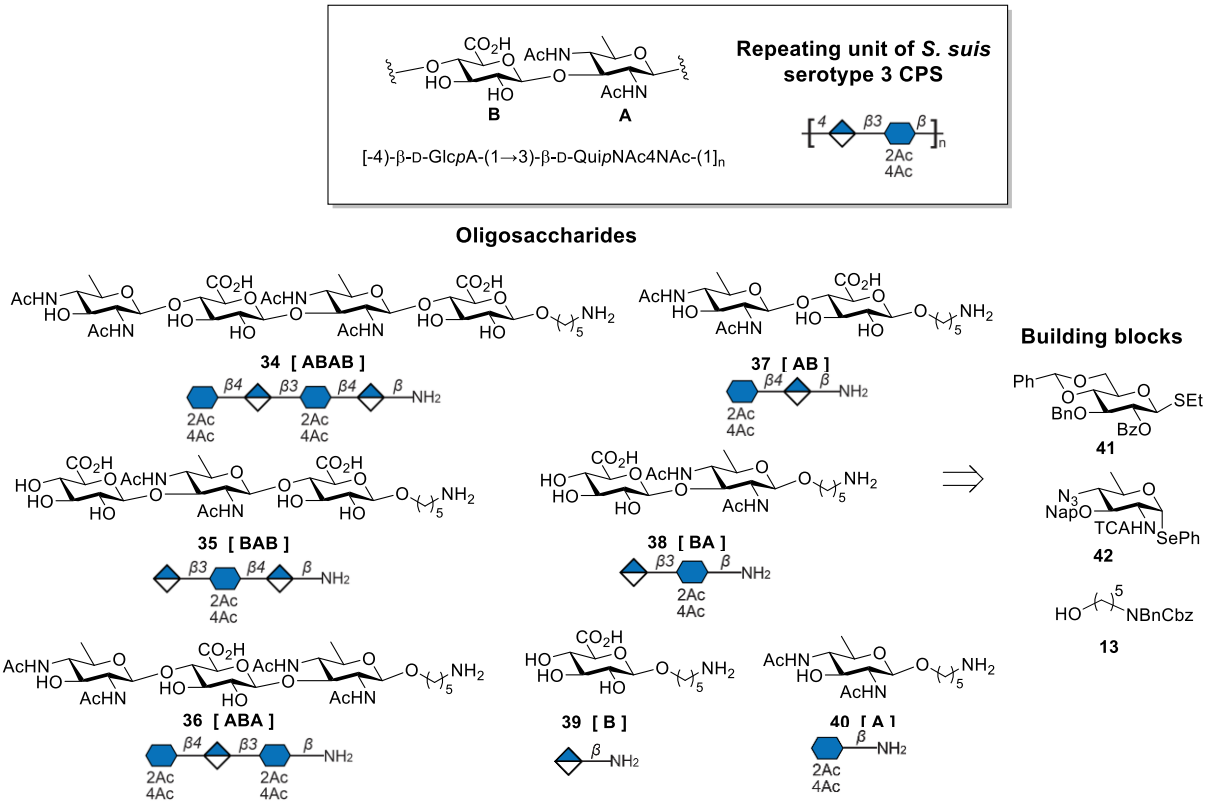

Figure 4. Structure of *S. suis* serotype **3** CPS and synthetic oligosaccharide antigens resembling the *S. suis* serotype 3 CPS derived from building blocks **13**, **41**, and **42**.

The main synthetic challenges are access to the rare diamino bacillosamine building block **42** and complex functional interconversions to be performed at the tri- and tetrasaccharide level. Formation of glycosidic bonds to construct the oligosaccharides was challenging as several electron-withdrawing groups reduce the nucleophilicity of glycosyl acceptors.[40,41] Since electron-withdrawing carboxyl groups at C-6 may further reduce the reactivity of the glucose donors,[42] they were introduced after the glycosylation. Finally, the densely nitrogen-functionalized target molecules necessitated additional deprotection steps. Stereoselective *β*-glycosidic bond formations were ensured by neighboring participation with benzoyl groups (Bz) on the glucose and a trichoroacetamide (TCA) group on bacillosamine.

Synthesis of the bacillosamine-containing reducing end commenced with the stereoselective glycosylation between selenoglycoside **42** (Supporting information, Scheme S5) and protected amino linker **13** using TMSOTf and NIS as activators to yield exclusively the *β*-linked product **43** in 91% yield. Conversion of the azide group to the acetamide and contemporary reduction of the TCA group was carried out using zinc powder in a THF/Ac_2O/AcOH mixture. Removal of the remaining benzyl ethers and the benzyloxycarbamate group was achieved by

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hydrogenolysis using Pd/C in EtOAc/*t*-BuOH/H_2O and gave linker-equipped bacillosamine **40** in 49% yield over two steps.

Cleavage of the 2-naphthylmethyl (Nap) protecting group on **43** with DDQ afforded alcohol **44** in 97% yield and subsequent uion of **44** and **41**[43] yielded 67% β-linked disaccharide **45**. Even when excess donor **41** (2.0 eq) and prolonged reaction times were employed, unreacted acceptor **44** was always observed. Acid hydrolysis of the benzylidene acetal on **45**, followed by selective oxidation of the C-6 hydroxyl group with TEMPO/BAIB and protection of the carboxyl moiety as benzyl ester gave disaccharide **47** in 77% yield over two steps. The C-4 hydroxyl was glycosylated with another bacillosamine unit to furnish β-linked trisaccharide **48** in 65% yield. Azide and TCA groups present in **47** and **48** were converted into acetamides by employing the same conditions used for compound **43**. Subsequent hydrolysis under basic conditions and hydrogenolysis smoothly afforded oligosaccharides **38** and **36** in acceptable yield.

Linker-equipped glucuronic acid was prepared by first coupling protected glucose building block **41** and **13** under NIS/TMSOTf promotion to furnish β-linked product **49** in 71% yield. The benzylidene acetal on **49** was then hydrolyzed, before regioselective oxidation with TEMPO/BAIB and esterification furnished glucuronic acid benzyl ester **51** in 72% yield over two steps. Ester groups on monosaccharide **51** were hydrolyzed under basic conditions, and hydrogenolysis afforded deprotected **39** in 70% yield over two steps.

Further elaboration of **51** continued with a glycosylation using building block **42** to furnish protected disaccharide **52** in 93% yield. The Nap ether was removed as preparation for another glycosylation with building block **41** to yield protected trisaccharide **54**. Removal of the benzylidene acetal on **54** proved challenging. Treatment with (+)-camphor-10-sulfonic acid as a catalyst and ethanethiol as acetal exchange reagent[44,45] gave trisaccharide diol **55** in low yield. Instead, aqueous acetic acid (80%) at 60 °C smoothly delivered **55** in 87% yield (Supporting information). The primary C-6 alcohol of diol **55** was then oxidized and converted into the corresponding benzyl ester **56**. Coupling of bacillosamine donor **42** with acceptor **56** produced tetrasaccharide **57** in 65% yield. Finally, oligosaccharides **52**, **56** and **57** were globally deprotected via Zn-mediated reduction, basic ester hydrolysis and hydrogenolysis, to obtain oligosaccharides **37** (51% over three steps), **35** (50% over three steps) and **34** (40% over three steps), respectively.

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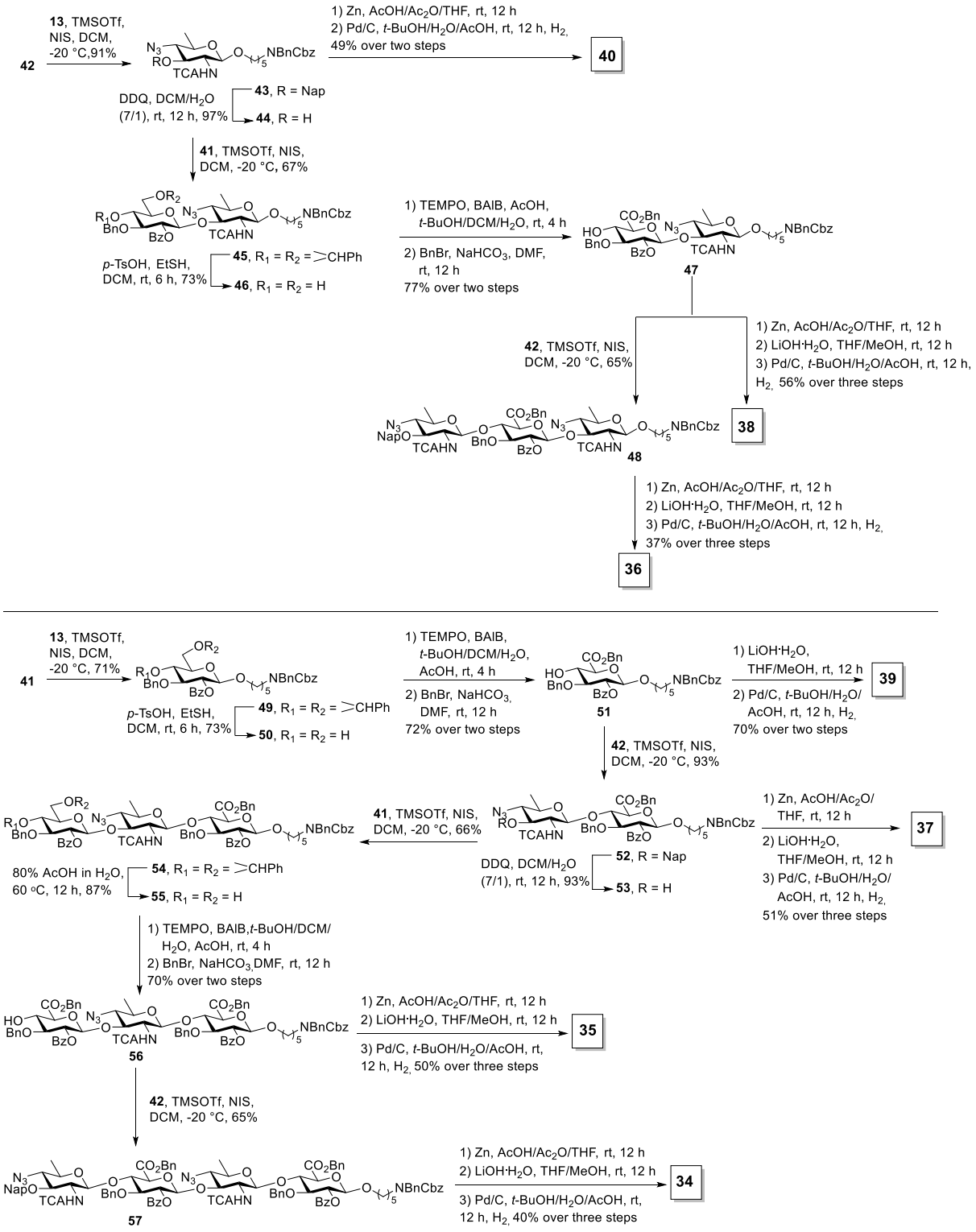

Scheme 3. Synthesis of oligosaccharides **34**-**40** related to *S. suis* serotype 3 CPS

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Synthesis of oligosaccharides related to *S. suis* serotype 9 CPS

The *S. suis* serotype 9 CPS repeating unit consists of a branched tetrasaccharide with a phosphorylated D-glucitol residue (Figure 5).[15] The presence of the labile C-4-keto sugar and the phosphodiester group pose considerable synthetic challenges. Synthetic C-4-keto sugars are difficult to handle during a total synthesis but the reduced form is more stable, while maintaining the ability to induce an immune response against the CPS.[46] This isosteric chemical modification facilitates subsequent scale-up. We focused on the synthesis of the C-4-axial reduced form of *S. suis* serotype 9 CPS repeating unit and its analogues (Figure 5).

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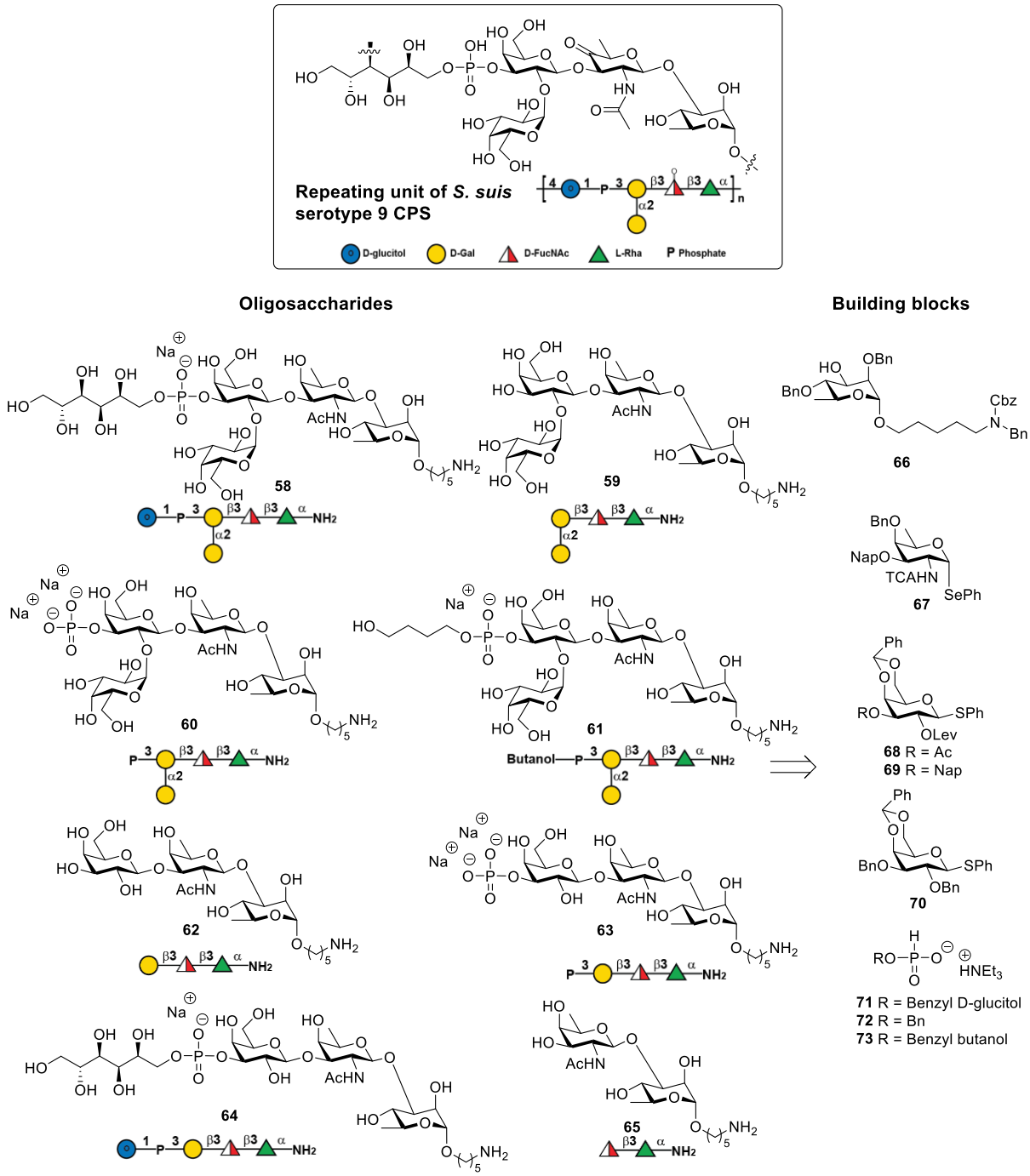

Figure 5. Structure of *S. suis* serotype 9 CPS and related oligosaccharides **58-65** to be prepared from building blocks **66-73**.

Five differentially protected monosaccharide building blocks (**66-70**) and three *H*-phosphonates (**71, 72, 73**) are needed to assemble the target oligosaccharides (Figure 5). Benzyl ethers serve as permanent protecting groups while TCA and levulinoyl ester (Lev) ensure anchimeric assistance as C-2 protective groups. The phosphorylated D-glucitol will be introduced after assembly of the tetrasaccharide core.

Assembly of tetrasaccharide **79** commenced with the union of L-rhamnose **66** and D-fucosamine **67** in the presence of NIS/TfOH as promoter to afford disaccharide **74** in good stereoselectivity

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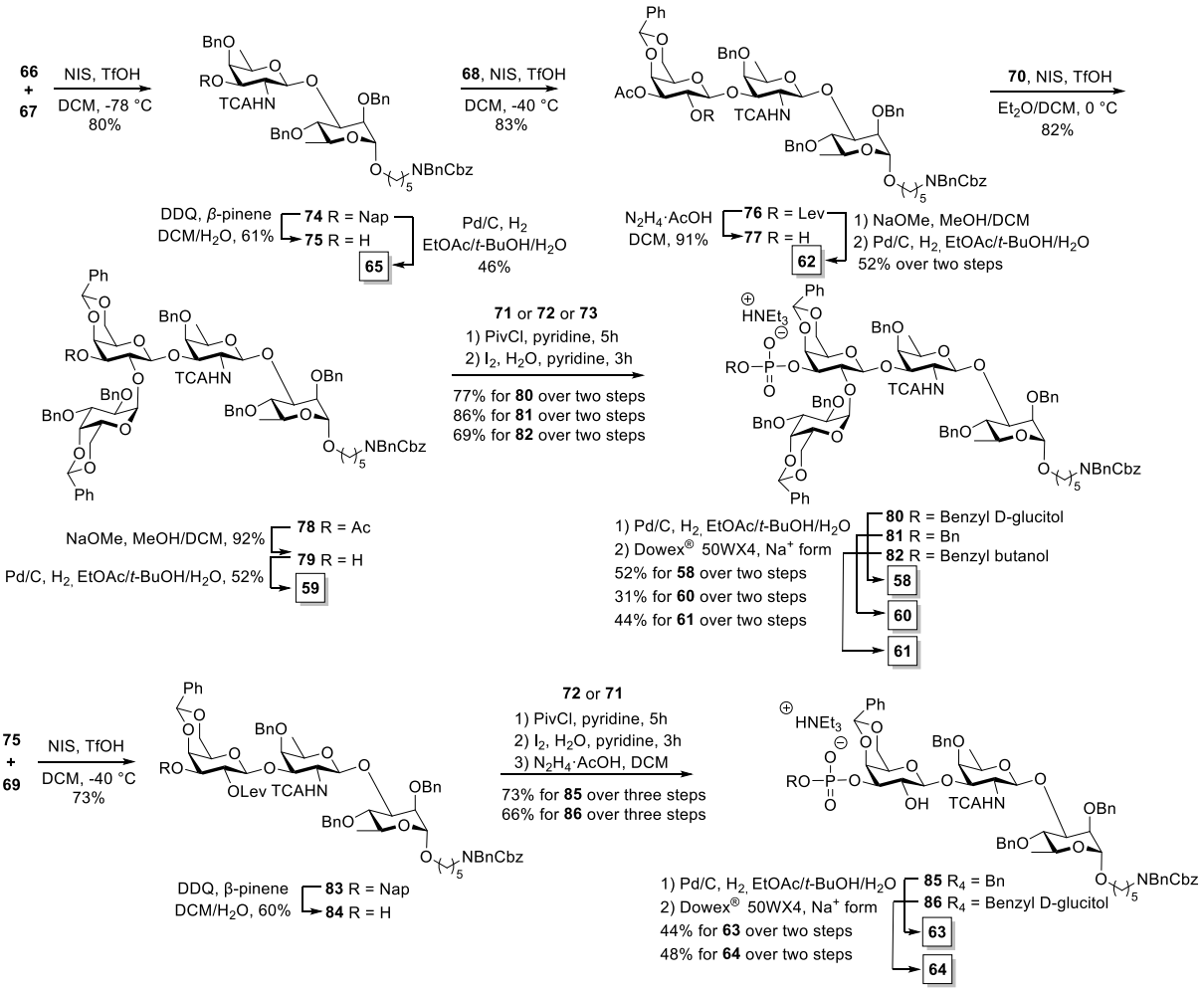
(α/β = 1/6) (Scheme 4). Cleavage of the Nap ether with DDQ and β-pinene gave disaccharide **75** (61% yield)[47] ready for glycosylation with **68** to produce trisaccharide **76** with complete β-selectivity. Cleavage of the levulinoyl ester with hydrazine acetate gave trisaccharide **77** in 91% yield. Control over stereoselectivity via solvent effects yielded consistant results. The NIS/TfOH-mediated glycosylation between thioglycoside **70** and **77** in a mixture of DCM and Et_2O produced 82% tetrasaccharide **78**. Acetyl ester cleavage with sodium methoxide furnished **79**. Global deprotection of oligosaccharides **74**, **76** and **79** gave the corresponding non-phosphorylated compounds **65**, **62** and **59**. *H*-phosphonates **71**, **72** and **73** were coupled with the hydroxyl group on **79** in the presence of pivaloyl chloride to give intermediate *H*-phosphonate diesters.[48] Subsequent oxidation with iodine and water in the same pot furnished the phosphates **80**, **81** and **82** as triethylammonium salts in 69-86% yield over two steps. Global deprotection of the phosphodiesters by Pd/C-catalyzed hydrogenolysis, followed by ion exchange column chromatography furnished **58**, **60** and **61** as sodium salts (Supporting information).



Scheme 4. Synthesis of oligosaccharide fragments of *S. suis* serotype 9 CPS **58-65.**

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For the synthesis of trisaccharide phosphates, glycosylation between **75** and **69** yielded trisaccharide **83** (73%) with complete *β* selectivity (Scheme 4). Removal of Nap provided **84** with a free C-3 hydroxyl group to be coupled with *H*-phosphonates **72** and **71**. The levulinoyl esters were cleaved to afford phosphate diesters **85** (73% yield) and **86** (66% yield). Target phosphates **63** and **64** were obtained after global deprotection.

The antibody-binding activity of a synthetic oligosaccharide antigen depends on the structure and configuration of the antigen.[49] In order to investigate the importance of the *β*-linkage in the *S. suis* serotype 9 CPS, oligosaccharides **87-93** bearing an *α*-glycosidic linkage in place of the native *β*-glycosidic bond between D-fucosamine and L-rhamnose based on the *S. suis* serotype 9 CPS were prepared (Figure 6).

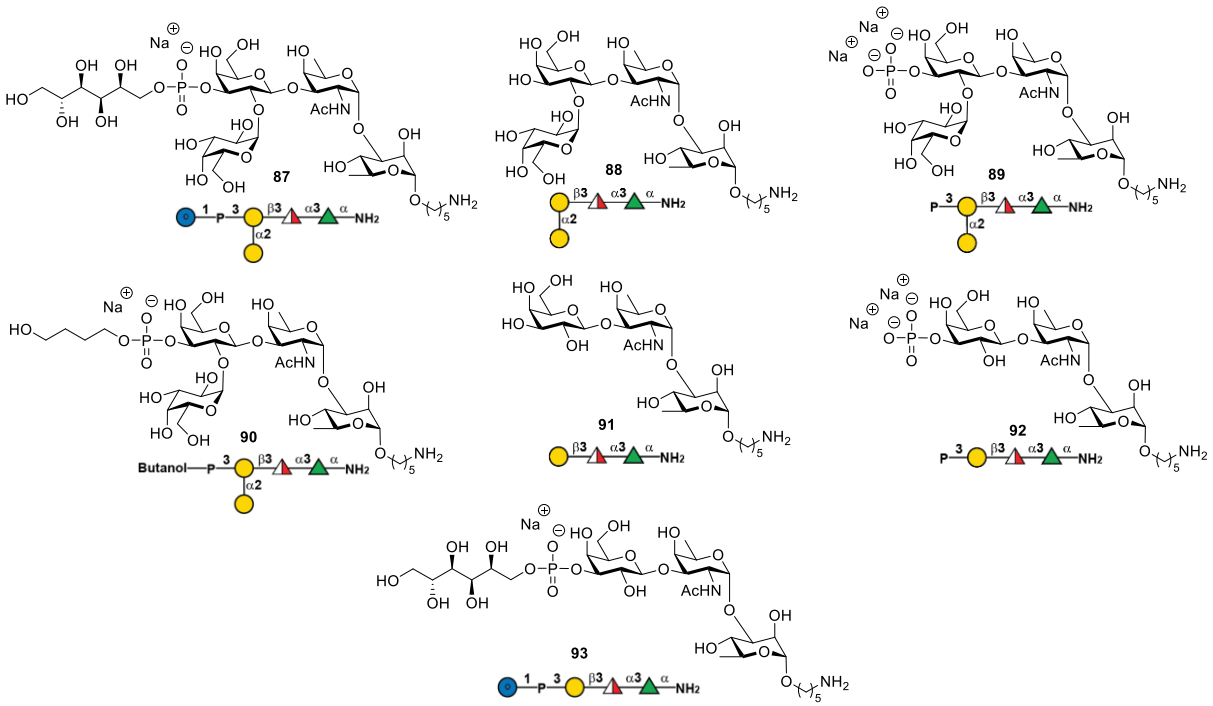

Figure 6. *α* Oligosaccharide antigens resembling *S. suis* serotype 9 CPS.

To install the *α*-linkage, building block **94** was prepared (Supporting information). Following the assembly sequence used for the *β*-linked oligosaccharides, union of **66** and **94** at 0 °C produced disaccharide **95** with high *α*-selectivity (Scheme 5). Conversion of the benzoyl ester to the benzyl ether over two steps followed by the cleavage of the Nap ether afforded disaccharide acceptor **98**. Coupling of thioglycoside **68** with **98** produced exclusively *β*-product **99** in 87% yield. After levulinoyl ester cleavage, NIS/TfOH-mediated glycosylation of **100** with **70** produced tetrasaccharide **101**. The azide moiety of **101** was reduced by zinc and the resulting amine was acetylated to give **102** in 76% yield. Next, tetrasaccharide **103** with a free hydroxyl was obtained after saponification of the acetyl ester and following hydrogenolysis gave **88**. Tetrasaccharide **103** was smoothly coupled with three different *H*-phosphonates **71**, **72** and **73**

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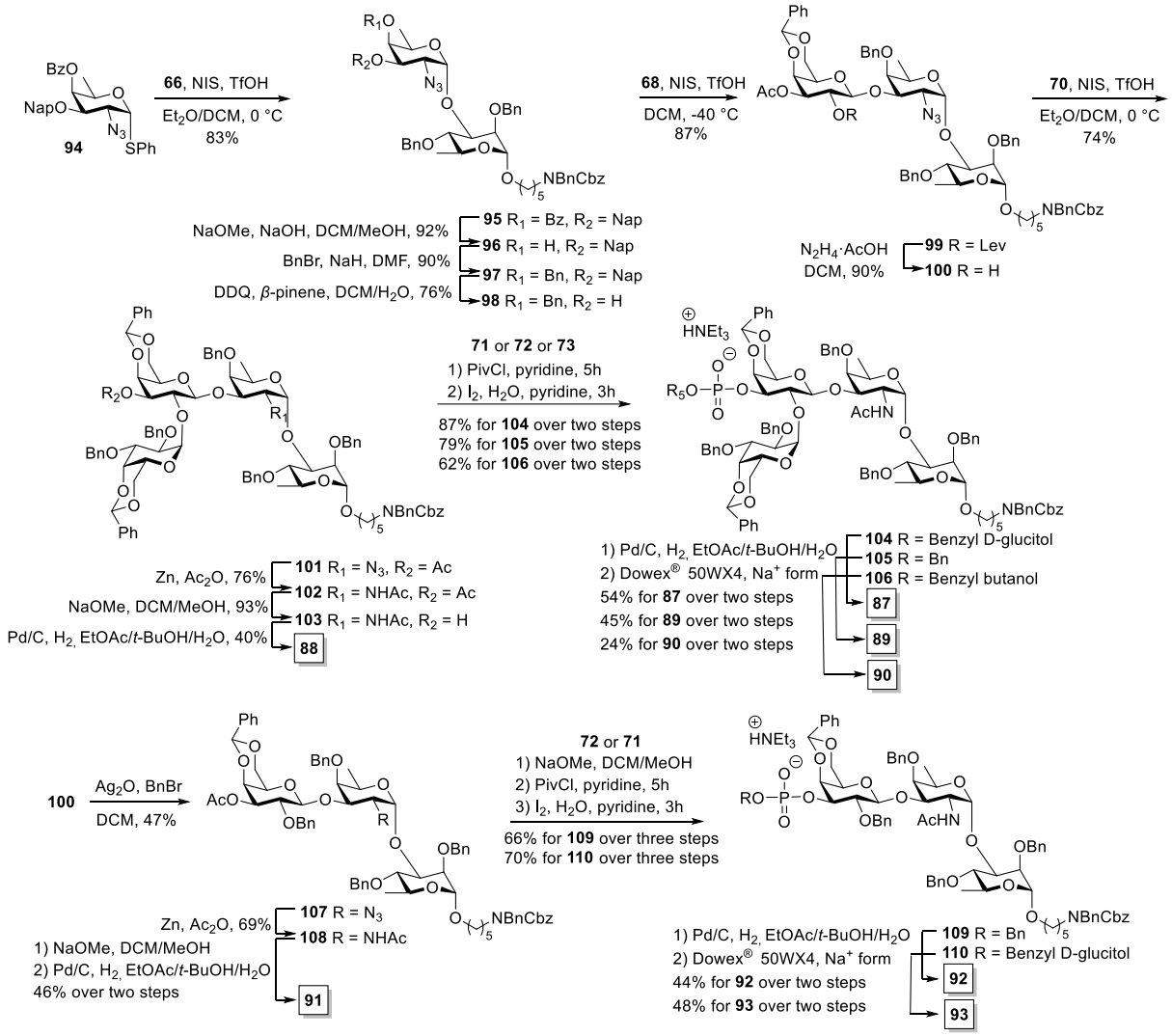
to produce corresponding phosphates **104**, **105** and **106**. Subsequent hydrogenolysis and sodium ion exchange chromatography produced pure **87**, **89** and **90**.



Scheme 5. Assembly of α oligosaccharides **87-93**.

The presence of an acetyl ester in **100** rendered benzylation under basic conditions not feasible. Treatment of **100** with freshly prepared silver oxide and benzyl bromide[50] produced **107** in 47% yield. The azide was converted to the corresponding acetamide using zinc and acetic anhydride to yield **108** (69%). The synthesis of **91** was achieved after removal of the acetyl group and hydrogenolysis of the benzyl ethers on **108** in 46% yield over two steps. To set the stage for phosphorylation, deacetylation of **108** yielded the requisite free hydroxyl group that was coupled with **72** and **71** to furnish phosphates **109** and **110** as the triethylammonium salts. Global deprotection of the phosphates afforded the corresponding final glycosides **92** and **93**.

Glycan array screening identifies a trisaccharide as *S. suis* serotype 9 glycoconjugate vaccine lead

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Sera from pigs infected with *S. suis* serotype 9, from immunized rabbits and human reference serum 007sp[51] were screened for antibodies binding to synthetic oligosaccharides and isolated *S. suis* serotype 9 CPS using glycan microarrays (Figure 7). IgG antibodies present in pig sera bound weakly to oligosaccharides revealing a complex binding pattern. Rabbit sera on the other hand showed a clearer picture. Rabbit IgGs recognized strongly phosphorylated trisaccharides **63** and **64**, disaccharide **65**, and similarly the native CPS. Trisaccharide **63** includes both the sugar sequence of **65** and a terminal phosphate monoester. This functional group appears to be essential since the absence on phosphorylation on **62** strongly reduced binding. On the other hand, the glucitol chain on **64** did not show a significant effect. Branched oligosaccharides **58-61** were not bound specifically. These observations suggest that the minimal glycotope contains L-rhamnose, D-fucosamine and a phosphate moiety, indicating that trisaccharide **63** is the minimum glycotope useful to elicit an immune response.

Antibodies from 007sp serum bound to the longer oligosaccharides due to cross-reactivity with CPS *S. pneumoniae*, possibly because of the branched α-galactose.[35,52] It had been shown previously, that other *S. suis* serotypes cross-react with some serotypes of *S. pneumoniae*.[35,36] The IgG response was higher than that of IgM, as IgM antibodies showed low or no binding for most cases (Supporting information, Figure S4) indicating that isotype switching after infection in pigs or immunization in rabbits and humans was induced.

Oligosaccharides with an α- in place of a β-linkage between D-fucosamine and L-rhamnose (**87-93**) were found to be bound much weaker (Supporting information, Figure S5). This finding suggests that the β-linkage, present in the native CPS,[15] has an important role for the recognition by antibodies.[49]

In conclusion, trisaccharide **63** is an attractive lead for the development of a glycoconjugate vaccine against *S. suis* serotype 9.

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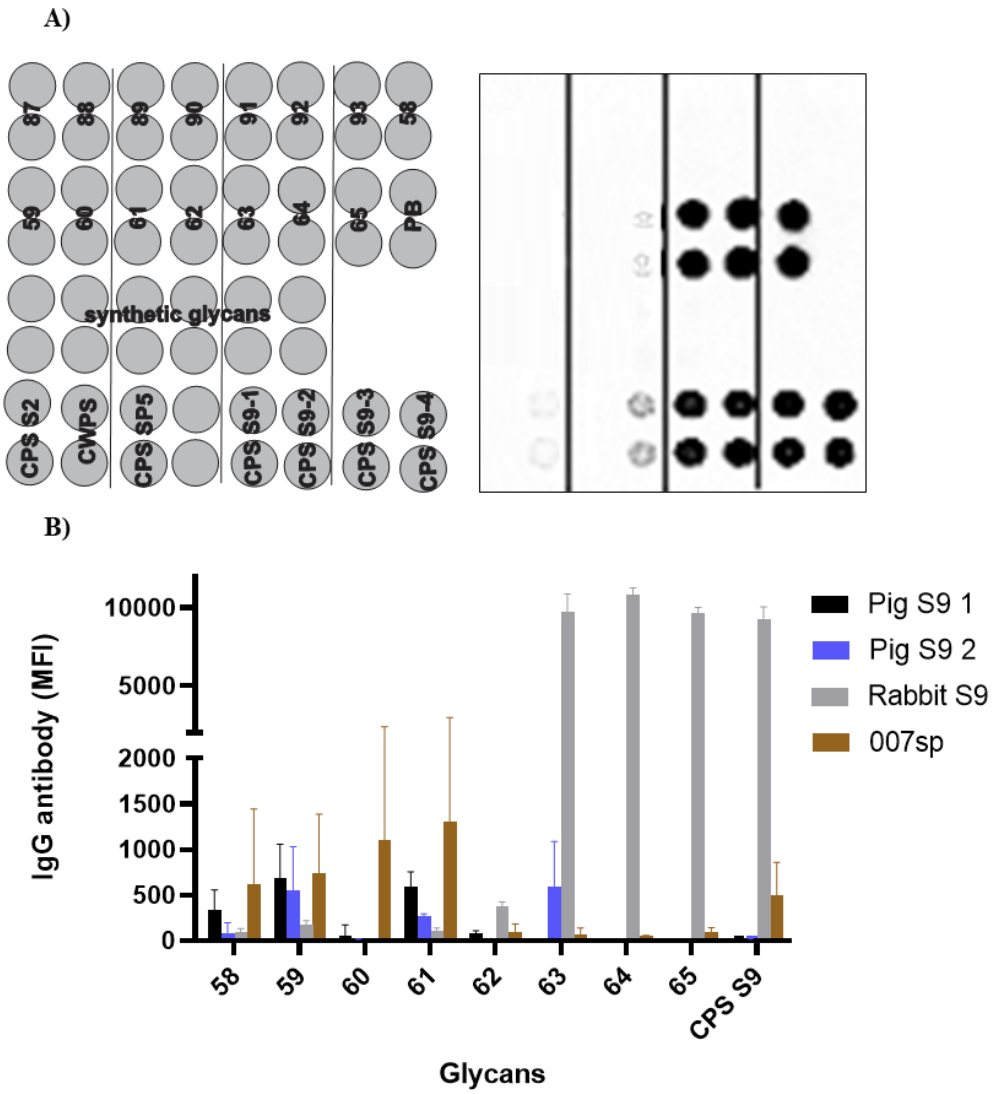

Figure 7. Glycan array analysis of *S. suis* serotype 9 oligosaccharides and native CPS. A) Printing pattern of microarray and binding of rabbit serum to immobilized glycans B) IgG antibody binding to glycans. A serum dilution of 1:100 was used. MFI, mean fluorescence intensity (mean ± standard deviation); PB, printing buffer; CWPS, cell wall polysaccharide; CPS and synthetic glycans, see Figure S3.

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Synthesis of oligosaccharides related to *S. suis* serotype 14 CPS

S. suis serotype 14 is responsible for pig and human infections mainly in Asia[6,53] and has been less studied than the more prevalent serotypes. Expression of the CPS is fundamental to inhibit phagocytosis *in vitro* and non-encapsulated bacteria are significantly less virulent in mouse models.[54] The serotype 14 CPS[13] (Figure 8) consists of a hexasaccharide repeating unit ([→6)[α-Neu5Ac(2→6)-β-D-Gal(1→4)-β-D-GlcNAc(1→3)]-β-D-Gal(1→3)-β-D-Gal(1→4)-β-D-Glc(1→]) composed of a trisaccharide backbone and a sialylated lactosamine side chain. Compared to the *S. suis* serotype 2 the β-rhamnose in the backbone is missing and the linkage between the glucose and galactose units is β–(1→6), instead of β–(1→4).

Recent studies aimed at elucidating glycotopes responsible for the production of protective antibodies[19] but more detailed information on the structure of carbohydrate epitopes is needed. Moreover, the antigenic properties of the CPS, either alone or as part of a glycoconjugate, have not been evaluated.

Several substructures related to the repeating unit of serotype 14 CPS were designed (Figure 8), including three oligosaccharides carrying an aminopentyl spacer at the reducing end. To identify whether antibody binding involves mostly the backbone residues,[19] hexasaccharide **111** was synthesized. Pentasaccharide **112** was prepared to evaluate whether antibody epitopes include the entire repeating unit. Hexasaccharide **113**, representing its sialylated analogue, was included to address the role of sialylation in *S. suis* serotype 14 CPS.

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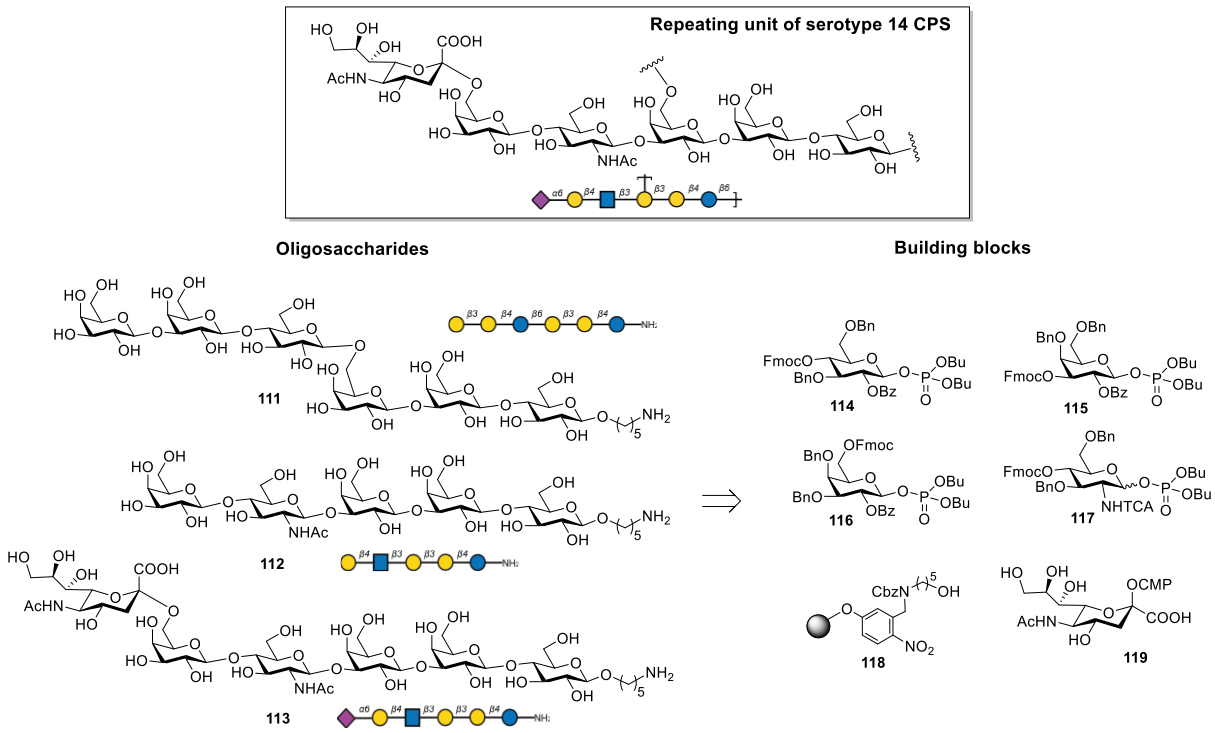

Figure 8. Structure of *S. suis* serotype 14 CPS and retrosynthetic analysis of related oligosaccharides. (CMP= cytidine monophosphate)

Automated glycan assembly (AGA) was used to assemble all oligosaccharides from four building blocks and Merrifield resin functionalized with a photolabile linker (Scheme 6). Considering the challenges encountered during sialylation of *S. suis* serotype 2 oligosaccharides, a chemoenzymatic approach was adopted to obtain hexasaccharide **113**, via one single step from pentasaccharide **112** using a sialyltransferase.

Hexasaccharide **111** consists of two repetitions of a trisaccharide. Glycosyl phosphates **114**, **115** and **116** were employed in AGA to obtain protected hexasaccharide **120**, using glycosylation conditions previously optimized for glycosyl phosphates. The target compound was obtained after resin cleavage and HPLC purification in 20% overall yield. Deprotection by hydrogenolysis and basic ester hydrolysis produced compound **111** in 52% yield over two steps. Pentasaccharide **112** contains the trisaccharide repeating unit in **111**, plus a glucosamine and a galactose. Just three building blocks are needed: one glucose, one galactose and galactosamine **117**. Employing the conditions used previously to synthesize the hexasaccharide, AGA proceeded smoothly and protected pentasaccharide **121** was obtained after resin cleavage and HPLC purification in 56% overall yield. Deprotection was carried out by hydrogenolysis followed by deacylation with sodium methoxide, to afford fully deprotected **112** in 53% yield. Enzymatic sialylation of pentasaccharide **112** using a sialyltransferase from the marine bacterium *Photobacterium damsela*[55] (Pd2,6ST) produced the $\alpha(2\rightarrow6)$ linkage in hexasaccharide **113**. To limit double sialylation on the oligosaccharide chain observed during

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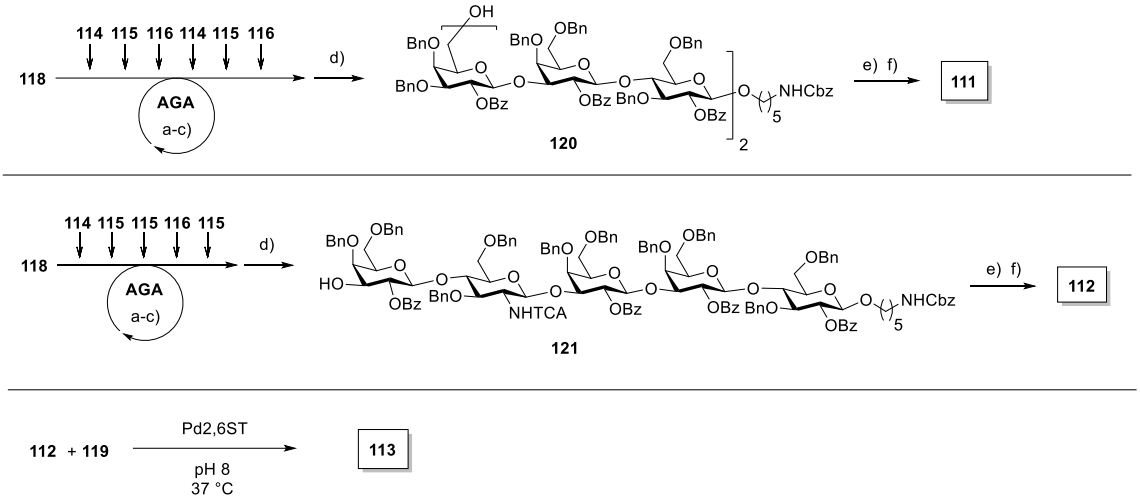
intial experiments, optimal reaction conditions (1.5 eq of CMP-Neu5Ac and 7 h reaction time) were used to produce sialylated hexasaccharide **113** in 42% yield after purification.



Scheme 6. Synthesis of *S. suis* serotype 14 related oligosaccharides. Reagents and conditions: a) Building block (5 eq), TMSOTf, −30 °C (5 min)→ −10 °C (40 min); b) Ac_2O, MsOH, DCM ; c) Piperidine, DMF; d) hν; overall yield (based on resin loading): 20% for **120**; 56% for **121**; e) H_2, Pd/C, THF/MeOH/AcOH; f) NaOMe, MeOH; 40 °C; 52% over two steps for **111**; 53% over two steps for **112**.

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Conclusions

We describe the synthesis of a collection of 30 novel oligosaccharides resembling the capsular polysaccharides related to four major serotypes (2, 3, 9, 14) of the bacterium *Streptococcus suis*. The syntheses tackled challenges associated with complex glycan targets, such as sialylation, the introduction of amino-rich sugars and labile phosphodiesters. The synthetic, conjugation-ready glycans were printed onto array surface to give rise to glycan microarrays. The glycan microarrays were used to screen the sera of pigs infected with different *S. suis* serotypes as well as sera from immunized rabbits. With the help of glycan array studies, the glycan epitopes of lead antigens for the development of semi-synthetic glycoconjugate vaccines to protect from *S. suis* serotypes 2 and 9 were identified. Currently, vaccination of pigs for challenge studies is being prepared in an effort to develop efficacious vaccines to protect pigs as well as people working in the swine industry while reducing the use of antibiotics.

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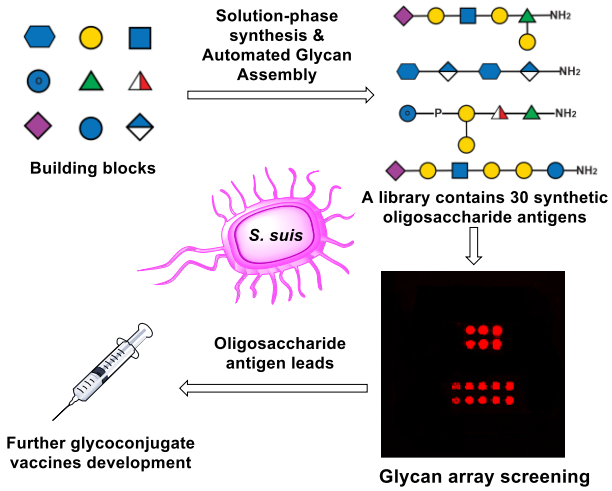

30 novel oligosaccharides resembling the capsular polysaccharides related to four major serotypes (2, 3, 9, 14) of *Streptococcus suis* (*S. suis*) were synthesized. The conjugation-ready glycans were used to screen the sera of pigs and rabbits. With glycan array study, glycan epitopes of lead antigens for the development of semi-synthetic glycoconjugate vaccines to protect from *S. suis* serotypes 2 and 9 were identified.

Keywords: Carbohydrates; Glycans; Immunology; Oligosaccharides; Total synthesis

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